



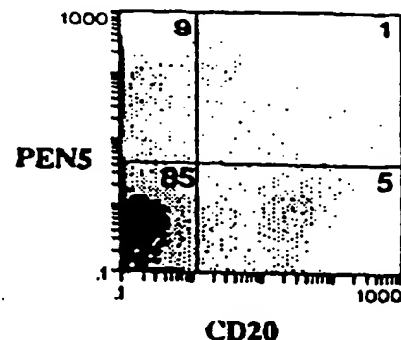
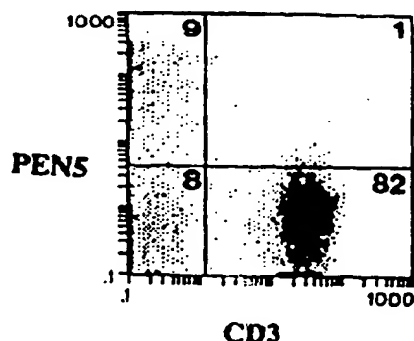
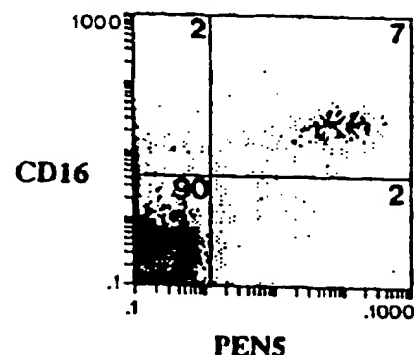
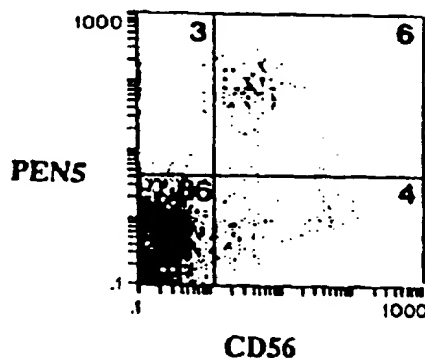
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| (21) International Application Number: PCT/US94/09714 (22) International Filing Date: 26 August 1994 (26.08.94) (30) Priority Data: 08/113,170 27 August 1993 (27.08.93) US (60) Parent Application or Grant (63) Related by Continuation US 08/113,170 (CIP) Filed on 27 August 1993 (27.08.93) (71) Applicant (for all designated States except US): DANA-FARBER CANCER INSTITUTE, INC. [US/US]; 44 Binney Street, Boston, MA 02115 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): ANDERSON, Paul [US/US]; 84 Duff Street, Watertown, MA 02172 (US). VIVIER, Eric [FR/FR]; 6, rue des Trois Frères Barthelemy, F-13288 Marseille (FR). (74) Agent: HART, Julia, D.; Dana-Farber Cancer Institute, Inc., 44 Binney Street, Boston, MA 02115 (US). | | (81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> |

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(54) Title: NATURAL KILLER CELL-SPECIFIC ANTIGEN AND ANTIBODIES THAT IDENTIFY THE SAME**(57) Abstract**

The invention provides a novel natural killer (NK) cell-specific molecule consisting of a glycoprotein pair PEN5-alpha and PEN5-beta, monoclonal antibodies and immunoreactive fragments and derivatives thereof that bind to unique epitopes present on this molecule, and hybridomas that produce the antibodies. Methods of using the antibodies, fragments, and derivatives are also provided. The PEN5-alpha and PEN5-beta glycoproteins have apparent molecular weights of 120-150 and 210-245 kilodaltons, respectively, as measured by SDS-PAGE on 6 % polyacrylamide gels. The unique epitopes of the glycoprotein pair are preferentially expressed on the subpopulation of peripheral blood NK cells having the phenotype CD16⁺ CD56^{dim} relative to their expression on peripheral blood NK cells having the phenotype CD16⁺ CD56^{bright}, and are not present on CD3⁺T lymphocytes or CD20⁺B lymphocytes. Figure 1 shows two-color flow cytometry histograms, which collectively reveal that the PEN5 epitope is expressed selectively on CD56⁺ CD16^{dim} peripheral blood lymphocytes.



**NATURAL KILLER CELL-SPECIFIC ANTIGEN AND ANTIBODIES
THAT IDENTIFY THE SAME**

5 Statement Of Government Rights In Invention

This work was supported by a National Institutes of Health grant, CA53595. The government of the United States of America has certain rights to this invention.

10 Cross Reference To Related Application

This application is a continuation-in-part of co-pending U.S. application Serial No. 08/113,170, filed August 27, 1993.

Field Of The Invention

15 The invention relates to novel cell surface structures that are selectively expressed on a subpopulation of natural killer cells and to antibodies that bind to unique epitopes on these structures.

20 Background Of The Invention

 Natural killer cells (hereinafter sometimes referred to as "NK cells") are large granular lymphocytes ("LGLs") comprising 2-15% of peripheral blood mononuclear cells in healthy individuals. Although NK cells do not rearrange or express either of the known T cell
25 receptor complexes, they can recognize and kill certain virus-infected and transformed cells in a non-MHC-restricted fashion, without prior sensitization. With the exception of CD16, an Fc receptor for immunoglobulin that recognizes antibody-coated target

structure selectively expressed on functionally differentiated NK cells.

Summary Of The Invention

5 These as well as other objects and advantages are achieved in accordance with the present invention, which provides a partially purified preparation of a novel natural killer cell-specific molecule, to monoclonal antibodies and immunoreactive fragments and derivatives thereof that bind to unique epitopes present on this NK
10 cell-specific molecule, and to hybridomas that produce the monoclonal antibodies. Methods of using the antibodies and fragments and derivatives are also provided.

 The novel NK cell-specific molecule of the invention consists essentially of a pair of polydispersed glycoproteins, designated
15 herein as PEN5 α and PEN5 β , having apparent molecular weights of 120 - 150 and 210 - 245 kdal, respectively, as determined by SDS polyacrylamide gel electrophoresis on a 6% polyacrylamide gel under non-reducing conditions. The unique epitopes of the PEN5 α /PEN5 β glycoprotein pair are preferentially expressed on the subpopulation
20 of peripheral blood NK cells which are of the phenotype CD16⁺ CD56^{dim} relative to their expression on peripheral blood NK cells having the phenotype CD16⁺ CD56^{bright} and are not present on CD3⁺ T lymphocytes or CD20⁺ B lymphocytes. In preferred embodiments of the invention, the antibody is unreactive with peripheral blood T
25 cells, activated T cells, thymocytes, peripheral blood B cells, splenic B cells, activated B cells, monocytes, granulocytes,

to herein as either anti-PEN5 or mAb 5H10, secreted by a hybridoma identified by ATCC Accession No. HB11441.

The antibodies and/or immunoreactive fragments or derivatives of the invention can be labeled, e.g. with a radioactive, enzymatic, or fluorescent label and used to detect, enumerate, and/or purify functionally differentiated NK cells in a mixed population of cells and to distinguish these cells from non-NK cells and NK cells that are not functionally differentiated. Identification of the functionally differentiated subpopulation of NK cells involves (a) contacting a suitable sample that contains a mixed population of cells, which can be, for example, peripheral blood, bone marrow aspirate, or lymphoid tissue, with an antibody of the invention or an immunoreactive fragment or derivative thereof, and (b) detecting immune complex formation. Immune complex formation can be detected by any of the techniques that are conventional and well known in the art.

The antibodies of the invention can also be used to selectively eliminate functionally differentiated NK cells that are of the phenotype CD16⁺ CD56^{dim} in a sample comprising a mixed population of cells. Thus, in another aspect of the invention, methods are provided for selectively eliminating or removing functionally differentiated natural killer cells from a suitable sample, preferably a biological sample, which involve (a) contacting the sample with an antibody of the invention or an immunoreactive fragment or derivative thereof, which is optionally linked to a radionucleotide or a toxin, and (b) removing from the

in each histograms indicate the percent of positive stained cells.

Figure 3 comprises a series of flow cytometry histograms illustrating the kinetics of PEN5 expression on activated NK cells. Sorted CD56^{dim} and CD56^{bright} NK cells were activated for 20 days with ionomycin and lymphocyte conditioned medium as described in the examples. At the indicated period of time, (i.e, 0, 6, 8, 10, 14, and 20 days of culture) aliquots of the activated NK cell populations were analyzed for their cell surface phenotype by flow cytometry using isotype matched control mAb, anti-CD56 and anti-PEN5 mAb. Results indicate the percent of positively stained cells (%); the total mean fluorescence intensity is indicated below.

Figure 4 is a series of flow cytometry histograms that illustrate the cell surface expression of the PEN5 epitope on leukemic NK cells. Peripheral blood NK cells, as well as peripheral blood mononuclear cells (PBMC) isolated from three patients undergoing granular lymphocyte proliferative disorder "GLPD" blast crisis (GLPD1-3) were analyzed for the cell surface expression of CD56 and PEN5 using indirect immunofluorescence and flow cytometry. The numbers in each histogram indicate the percent of positive stained cells.

Figure 5 is a reproduction of an SDS gel from an immunoprecipitation of PEN5 glycoproteins. Detergent lysates prepared from radioiodinated NK cells were immunoprecipitated using 5H10 mAb or mouse IgM control. Samples were then separated under non-reducing conditions on a 6% SDS-polyacrylamide gel.

glucose 6-phosphate, glucose and galactose.

In Figure 7B, peripheral blood NK cells were incubated in PBS-1%BSA for 3 hr with glycosidases (0.025 U/ml) or 45 min with proteases (5 mg/ml) at 37°C, respectively. Cell surface expression of the PEN5 epitope was then analyzed by flow cytometry using anti-PEN5 mAb. Percent modulation was calculated as the ratio of the total linear mean fluorescence intensity of the treated cells over that of untreated control cells.

In Figure 7C, the antigenicity of anti-PEN5 mAb for aggrecan proteoglycans was analyzed by ELISA as described in the Examples. The anti-keratan sulfate mAb 5D4 was used as a positive control. Chondroitinase ABC was used at 0.04 U/ml, keratanase I was used at 0.05 U/ml and keratanase II was used at 0.004 U/ml, for 1 hr at 37°C. In Figure 7C, the cross-hatched bars represent reactivity with the anti PEN5 antibody, 5H10, while the open bars represent reactivity with the control antibody, 5D4. Abbreviations used are: CD1 = embryonic chick cartilage aggrecan; BNC = bovine nasal cartilage aggrecan; RC = swarm rat chondrosarcoma aggrecan; and SHK = shark cranial cartilage aggrecan.

Figures 8A through 8C illustrate the results of immunogold staining of the PEN5 epitope on NK cells. Peripheral blood NK cells were stained with anti-PEN5 mAb followed by gold-labeled anti-mouse IgM antibodies, and glutaraldehyde-fixed cells were then analyzed by transmission electron microscopy. Magnification: x48500, 0.972 cm = 200 nm. The three photomicrographs 8A-8C represent different views of the same stained cell.

Figure 9 illustrates a comparative histochemical staining of normal adult lymph node and tonsil. Magnification of PEN5 staining shown in the far right panels is 40X. Magnification in all other panels is 10X. Monoclonal antibodies used to stain tissue sections, and specific methods are described in the Materials and

of both PEN5⁺ cells and TIA-1⁺ cells in panels B and D.

Detailed Description Of The Invention

Natural killer cells are CD3:TCR⁻, CD16⁺, CD56⁺ large granular lymphocytes. Two functionally distinct populations of peripheral blood NK cells can be differentiated by their surface expression of an isoform of the neural cell adhesion molecule, NCAM (also known as CD56). CD56^{bright} NK cells have the attributes of an undifferentiated cell in that they proliferate vigorously in response to exogenous cytokines, but largely lack cytolytic activity. CD56^{dim} NK cells have the attributes of a more differentiated cell, in that they proliferate poorly in response to exogenous cytokines, but are potent cytolytic effector cells. Several monoclonal antibodies that recognize human CD56 are available commercially, for example from Coulter Corp. (Hialeah, Florida) and AMAC, Inc. (Westbrook, Maine).

NK cells are capable of mediating two types of cytotoxic effector function: natural cytotoxicity and antibody-dependent cellular cytotoxicity ("ADCC"). In this capacity, NK cells play an important role in host defense against viral infection, and in immune surveillance against the establishment of transformed cells. More recent results indicate that NK cells can effect a primitive form of allorecognition which can contribute to graft rejection during allogeneic transplantation and also to graft-versus-host disease. For these reasons, the reliable identification of NK cells within the mononuclear cell population is of great importance.

CD20⁺ B cells. The "unique epitope" may be present on a glycosylated form of the PEN5 glycoprotein pair as it is ordinarily expressed on the cell surface of CD56^{d1a}, CD16⁺ natural killer cells as previously described, or, the "unique epitope" may be present on an unglycosylated or deglycosylated form of the PEN5 glycoprotein pair. As used herein, the term "unglycosylated" means a PEN5 molecule where both the PEN5 α and the PEN5 β glycoproteins are free of any covalently attached carbohydrate moieties. As used herein, the term "deglycosylated" means a PEN5 molecule where either or both of the PEN5 α glycoprotein or the PEN5 β glycoprotein is partially glycosylated but does not contain the same full contingent of carbohydrate moieties as the PEN5 α glycoprotein or the PEN5 β glycoprotein as it is ordinarily expressed on the cell surface of CD56^{d1a}, CD16⁺ natural killer cells.

The antibodies, fragments, and derivatives of the invention are useful as research reagents, to unambiguously identify, quantify and/or purify natural killer cells in a mixed population of cells and in isolating these natural killer cells therefrom. The antibodies, fragments and derivatives of the invention may also be useful therapeutically, either alone, in combination with complement, or conjugated to a radioactive material or a toxin to treat disorders of the immune system where NK cells are implicated as mediators of disease, especially graft-versus-host disease and solid organ and allogenic bone marrow transplant rejection. Monoclonal antibodies, and chimeric and humanized antibodies, are preferred for detection and therapy, respectively. Antibodies,

sufficient time, the animal is sacrificed and spleen or other immune cells obtained. The preferred immunogen to be used in the immunization protocol is a preparation of freshly isolated NK cells, purified from peripheral blood lymphocytes by negative selection. Other immunogens that alternatively could be used include partially purified preparations of the PEN5 molecule, the PEN5 α glycoprotein or the PEN5 β glycoprotein, including the glycosylated, deglycosylated or unglycosylated forms thereof and derivatives and fragments thereof. A partially purified preparation of the PEN5 molecule can be prepared from permeabilized NK cells following immunoprecipitation and SDS gel electrophoresis using 6% polyacrylamide gel as hereinafter described using techniques well known to persons skilled in the art. However, any suitable method for partially purifying the PEN5 molecule or the PEN5 α or the PEN5 β glycoprotein as described above can be satisfactorily employed and alternative methods of partial purification will be readily apparent to those persons skilled in this area of technology. Once the protein core(s) of the PEN5 α and PEN5 β molecules have been cloned, recombinantly produced molecules can also be used as an immunogen. The spleen or other immune cells obtained from the animal are immortalized by fusing the spleen cells with an immortalized cell line, generally in the presence of a fusion enhancing reagent, for example, polyethylene glycol. The resulting cells, which include the fused hybridomas, are then allowed to grow in a selective medium, such as HAT medium, and the surviving cells are grown in such medium using limiting dilution

phenotypically CD16⁺ CD56^{bright} can be conducted by testing on purified populations of lymphoid and non-lymphoid cells by indirect immunofluorescence assays and flow cytometry, substantially as described in the Examples herein. Monoclonal antibodies that recognize a PEN5 epitope that is preferentially expressed on functionally differentiated NK cells will react with an epitope that is present on a high percentage NK cells that phenotypically are CD56^{dim} CD16⁺ cells, e.g., at least about 70 - 90%, preferably about 80%, of such cells, and with a much lower percentage of NK cells that are phenotypically CD16⁺ CD56^{bright} (e.g., about 10 to 35%), but will not react with CD3⁺ T cells or CD20⁺ B cells. In preferred embodiments, the antibody will also be unreactive with monocytes, granulocytes, platelets, and red blood cells. Monoclonal antibodies that compete with the 5H10 antibody in competition assays well known to persons skilled in the art are likely to recognize essentially the same epitope as mAb 5H10, while monoclonal antibodies that fail to compete with mAb 5H10 but nevertheless meet the criteria of being unique to the CD16⁺, CD56^{dim} subpopulation of NK cells are likely to recognize a different epitope on the PEN5 glycoprotein pair. Both classes of antibodies are considered within the scope of the present invention.

Once the desired hybridoma has been selected and cloned, the resultant antibody may be produced in one of two major ways. The purest monoclonal antibody is produced by in vitro culturing of the desired hybridoma in a suitable medium for a suitable length of time, followed by the recovery of the desired antibody from the

antibodies exhibiting the characteristics of mAb 5H10 as herein described.

For example, it was determined that the subject monoclonal antibody 5H10 belongs to the class IgM. However, a monoclonal antibody exhibiting the characteristic described herein may be of class IgG, subclass IgG₁, IgG₂ α , IgG₂ β , or IgG₃, or of classes IgM, IgA, or other known Ig classes. The differences among these classes or subclasses will not affect the selectivity of the reaction pattern of the antibody, but may affect the further reaction of the antibody with other materials, such as (for example) complement or anti-mouse antibodies. Although the subject antibody is specifically IgM, it is contemplated that antibodies having the patterns of reactivity illustrated herein are included within the subject invention regardless of the immunoglobulin class or subclass to which they belong.

Moreover, while the specific example of the novel antibody of the present invention is from a murine source, this is not meant to be a limitation. The above antibody and those antibodies having the characteristics of the mAb 5H10, whether from a mouse source, other mammalian source including human, rat, or other sources, or combinations thereof, are included within the scope of this invention, as set forth above.

The antibodies may be used for the detection and enumeration by indirect staining of CD16⁺, CD56^{d1a} subpopulation of NK cells in normal individuals or in disease states, for example by fluorescence microscopy, flow cytometry, immunoperoxidase, or other

One approach for minimizing or eliminating this problem, which is preferable to general immunosuppression, is to produce chimeric antibody derivatives, i.e. antibody molecules that combine a non-human animal variable region and a human constant region. Chimeric antibody molecules can include, for example, the antigen binding domain from an antibody of a mouse, rat, or other species, with human constant regions. A variety of approaches for making chimeric antibodies have been described and can be used to make chimeric antibodies containing the immunoglobulin variable region which recognize a unique epitope on the PEN5 antigen. See, for example, Morrison et al., Proc. Natl. Acad. Sci. U.S.A. 81:6851 (1985); Takeda et al., Nature 314:452 (1985), Cabilly et al., U.S. Patent No. 4,816,567; Boss et al. U.S. Patent No. 4,816,397; Tanaguchi et al., Eur. Patent Pub. EP171496; Eur. Patent Pub. 0173494; United Kingdom Patent GB 2177096B. Such chimeras produce a less marked immune response than non-chimeric antibodies.

For human therapeutic purposes, the monoclonal or chimeric antibodies of the invention can be further humanized by producing human constant region chimeras, in which even parts of the variable regions, especially the conserved or framework regions of the antigen-binding domain, are of human origin and only the hypervariable regions are of non-human origin. Such altered immunoglobulin molecules may be made by any of several techniques known in the art, (e.g., Teng et al., Proc. Natl. Acad. Sci. U.S.A., 80:7308-7312 (1983); Kozbor et al., Immunology Today, 4:7279 (1983); Olsson et al., Meth. Enzymol., 92:3-16 (1982)), and

a radionucleotide or a toxin, such as diphtheria toxin, in conjugated form is thus targeted to the implicated NK cells.

Immunoassays

5 The antibodies of the invention and the fragments and derivatives thereof containing the binding region (e.g., Fab, Fab', F(ab')₂), can be used in various immunoassays. Such immunoassays include, but are not limited to, competitive and non-competitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme
10 linked immunosorbent assay), "sandwich" immunoassays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, and immunoelectrophoresis assays, to name but a few.

15 Differentiated NK cells, especially human NK cells, can be detected in a biological sample including a mixed population of cells, for example, hematopoietic and lymphoid cells, using the antibodies, fragments or derivatives. Suitable biological samples include peripheral blood, bone marrow aspirate and lymphoid tissue.
20 When used in an assay as described, the antibody is typically labeled so that its binding with the relevant NK cell subpopulation can be detected. Any suitable label well known to persons skilled in the art, including but not limited to fluorescent dyes, radioactive isotopes, enzymes which catalyze a reaction producing
25 detectable products, biotin, or metal ions detectable by nuclear magnetic resonance can be employed.

213 (1992) and Moretta et al, Immunol. Today, 13:300-305 (1992).

The scientific literature also suggests that NK cells may play a deleterious role in graft-versus-host disease ("GVHD") following solid organ or tissue transplants. (Ferrara et al, Transplantation, 47:50-54 (January, 1989); MacDonald and Gartner, Transplantation, 54:147-151 (July, 1992)). See also, U.S. Patent No. 4,772,552.

Since the antibodies of the invention can be used to target the functionally differentiated subpopulation of NK cells specifically, the invention may also be useful prophylactically and therapeutically, in the prevention and treatment of graft rejection in solid organ and bone marrow transplantation, and in graft-versus-host disease, by modulating the function and number of cytolytic effector NK cells in vivo. Although it is contemplated that the anti-NK cell-specific antibodies and fragments and derivatives thereof will have applicability for animal subjects in addition to human beings, such as domesticated animals, the therapeutic aspects of the invention are of the greatest value in the treatment of disorders in humans.

For example, in bone marrow transplantation, the antibodies, fragments and derivatives of the invention can be used to remove the CD16⁺ CD56^{dim} cytolytic effector population of cells from bone marrow aspirates ex vivo, prior to transplantation of the marrow into the marrow recipient. Removal of these natural killer cells from the bone marrow aspirate can be accomplished by conventional methods, such as those used in immunological T cell depletion. Antibodies that exhibit the ability to lyse NK cells in the

various intracellular lesions, well known to persons skilled in the art of radiotherapy.

Cytotoxic drugs that can be conjugated to antibodies and administered for in vivo therapy include, but are not limited to, daunorubicin, doxorubicin, methotrexate, and mytomicin C. For a more detailed discussion of these classes of drugs and their mechanisms of action, see, Goodman et al., Goodman and Gilman's The Pharmaceutical Basis Of Therapeutics, 8th ed. Pergamon Press (1991).

As an example of conjugation to a toxin, an anti-PEN5 monoclonal antibody can be combined with diphtheria toxin, by the method of Bumol, Proc. Natl. Acad. Sci., 80:529 (1983). Briefly, monoclonal antibodies reactive with an NK cell specific epitope are prepared as described by Bumol. The antibodies are purified and combined with excess (6 mol/mol) N-succinimydyl 3-(2-pyridyldithio) propionate (Pharmacia, Uppsala, Sweden) in PBS. After 30 minutes incubation at room temperature, the solution is dialyzed against PBS. The modified antibodies are conjugated with an appropriate toxin, such as diphtheria toxin A chain. Other toxins such as ricin A can also be employed. The diphtheria toxin A chain is isolated as detailed in Bumol, supra. The modified antibodies are mixed with excess (3 mol/mol) reduced diphtheria toxin A chain (10% of the total volume), allowed to react for 36 hours at 4°C, and concentrated by chromatography on Sephadex G-2000. The product is applied to a Sephadex G200 column (1.0 x 100 cm), allowed to equilibrate and eluted with PBS.

that increase the stability or half life of the antibody. To extend the half-life, the reagent can first be modified to increase or decrease the amount of carbohydrate complexed to it, or alternatively, can be complexed with a reagent such as polyethylene glycol. Finally, pharmaceutical compositions comprising the therapeutic reagent in the appropriate buffers, salts, and pH are required.

Therapeutic kits can comprise the therapeutic compositions of the invention in one or more containers.

The PEN5 α /PEN5 β Glycoprotein Pair

The invention also provides partially purified preparations of the NK cell-specific molecule, called PEN5 α /PEN5 β , that is preferentially expressed on the subpopulation of NK cells previously characterized as CD16⁺, CD56^{din} NK cells. The molecule consists essentially of two membrane bound glycoproteins.

As used herein, use of the term "partially purified preparation" with respect to the PEN5 means the PEN5 molecule, consisting essentially of the PEN5 α and PEN5 β glycoprotein pair as herein described, which has been purified from permeabilized NK cells following immunoprecipitation and SDS gel electrophoresis using 6% polyacrylamide gel as hereinafter described. After the glycoproteins are fractionated on a gel, they can be recovered and renatured in accordance with known and established techniques.

As expected of a marker of functional differentiation, expression of the PEN5 epitope is down-modulated by stimuli which induce NK

proteoglycans.

Two types of keratan sulfate proteoglycans have been described: cartilage-type keratan sulfate proteoglycans are O-linked glycoproteins, whereas cornea-type keratan sulfate proteoglycans are N-linked glycoproteins. Since PEN5 α is an N-linked glycoprotein, it is possible that PEN5 α is an unusual cell surface cornea-type keratan sulfate proteoglycan. Similarly, PEN5 β is an O-linked glycoprotein sensitive to keratanase treatment, and may be a cartilage-type keratan sulfate proteoglycan. However, the inability of six distinct anti-keratan sulfate mAbs to bind to NK cells, coupled with the lack of detection of S³⁵sulfur-labeled material in 5H10 (anti-PEN5) immunoprecipitates prepared from S³⁵ sulfur metabolically-labeled NK cells, indicate that the PEN5 glycoproteins are not keratan sulfate proteoglycans.

Alternatively, it has been reported that mucin-type glycoproteins secreted by cultured hamster tracheal epithelial cells are sensitive to keratanase I treatment and contain polylactosamine carbohydrates [Wu, Biochem J., 277:713 (1991)]. Mucin-type glycoproteins are highly glycosylated proteins containing a majority of O-linked oligosaccharides, and are associated with the cell membrane in a number of cell types [Carraway, Glycobiology 1:131 (1991); Strous, Rev. Biochem Mol. Bio. 27:57 (1992); Devine, 35 (1992). Classification of PEN5 β as an NK cell specific membrane-bound mucin-type glycoprotein is most consistent with our data. By contrast, the high content of N-linked carbohydrates in PEN5 α is not consistent with its classification as a mucin-type

consistent with their acquisition of fully competent cytotoxic function. Exogenous mucins have been shown to inhibit NK cell killing, supporting their potential involvement in resistance to NK cell cytolytic functions [Ogata, Cancer Res., 52:4741 (1992)].

5 The PEN5 antigen can be used in preparing and/or purifying the antibodies of the invention and should also be useful in identifying the natural counter-receptor for the PEN5 antigen on target cells. Amino acid sequence information obtained from the PEN5 glycoprotein pair can also be used to clone the PEN5 α and
10 PEN5 β glycoprotein chains in accordance with established techniques.

Deposit Information

15 Samples of the hybridoma (designated herein as 5H10) that secretes anti-NK cell-specific mouse monoclonal mAb 5H10 were deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland on August 19, 1993 under the terms of the Budapest Treaty and assigned ATCC accession number HB11441. Without admitting that access to the hybrid cell line is necessary to practice the claimed invention, it is agreed that, upon
20 allowance and issuance of a patent for this invention, all restrictions on the availability of the culture deposit designated herein will be removed and the designated culture will be maintained throughout the effective life of the patent granted, for 30 years from the date of deposit or for five years after the last
25 request for the deposit after issuance of the patent, which ever is longer.

(keratan sulfate 1,4 b-D-galactanohydrolase; ICN Biomedicals, (Costa Mesa, CA), keratanase II which attacks oversulfated forms of keratan sulfate resistant to keratanase I (Seikagaku America, Rockville, MD) and neuraminidase (Calbiochem) were used in either
5 PBS, PNGase F or O-glycanase buffers. Chondroitinase ABC (ICN Biomedicals,) was used in sodium acetate 0.05M pH 7.4. Bovine cornea keratan sulfate (BC), as well as other glycosaminoglycans and carbohydrates were purchased from Sigma, (St. Louis, MO)., Trypsin, chymotrypsin and pronase E were also obtained from Sigma.
10 FITC- and PE-conjugated avidin were obtained from Becton-Dickinson, (Paramus, NJ).

Antibodies.

Mouse monoclonal antibodies (mAb) reactive with CD2 (T11.1, IgG1), CD3 (RW24B6, IgG2b), CD56 (N901, IgG1), CD20 (B1, IgG1) were
15 obtained from Coulter Corp., as well as isotype matched control mouse mAb (IgG and IgM). Radioiodination of PEN5 mAb was performed using Iodobeads (Pierce) as previously described [Vivier, J. Immunol. 132:1410 (1991)]. The characterization of the anti-CD16
20 mAb (3G8, IgG1), and the anti-keratan sulfate mAb 5D4 (IgM) was reported elsewhere [Perussia, J. Immunol., 134:1410 (1984), Caterson, J. Biol. Chem., 258:8848 (1983)]. The following mAb recognize distinct epitopes on most keratan sulfate chains: 1B4 (IgG), 2D3 (IgG), 3D2 (IgM), 4D1 (IgM) and 8C2 [Sorrell, J. Invest. Dermatol. 95:347 (1990)]. FITC-labeled goat anti-mouse Ig(G+M) was
25 purchased from Tago.

centrifugation at 12,000 rpm for 15 min, radioiodinated lysates were diluted in 1 ml lysis buffer and precleaned three times with 3 μ l of affinity-purified rabbit anti-mouse IgM or IgG (RAM, Jackson ImmunoResearch Laboratories, (West Grove, PA) and 50 ml of a 50% solution of protein A-sepharose beads (Pharmacia, Milwaukee, WI). The immunoprecipitations were performed using 3 ml of the indicated mAb, 3 μ l of RAM and 50 μ l of protein A-Sepharose beads at 50%. Sepharose-bound immune complexes were washed four times in lysis buffer, and eluted either directly into sample buffer (2% SDS, 10% glycerol, 0.1 M Tris-HCl, pH 6.8, 0.02% bromophenol blue) prior to electrophoretic separation, or in elution buffer (0.15 M NH_4OH , pH 10.5) prior to deglycosylation experiments.

Deglycosylation of radioiodinated PEN5.

Radioiodinated PEN5 samples eluted from 5H10-coated Sepharose beads, were dried under vacuum and resuspended in appropriate deglycosylation enzyme buffers. The following enzymes were used alone or in combination: PNGase F (310 U/ml), O-glycanase (0.06 U/ml), keratanase I (0.25 U/ml) and neuraminidase (0.2 U/ml).

ELISA for aggrecan-type proteoglycans.

Wells of microtiter plates were incubated with 10 μ g/ml solutions of the indicated aggrecan-type proteoglycans overnight at 4°C. After washing, wells were incubated with 0.1 M Tris, pH 7.6 containing 1% BSA or with the indicated enzymes in this buffer. Following enzymatic digestion, a standard ELISA was performed using

reactive with CD5 (24T6G12, IgG2A), CD3 (RW24B6, IgG1), CD20 (B1H299, IGG2A), CD24 (MY4322A-1, IgG2B) at optimal concentrations for thirty minutes, then washed extensively. Following the addition of magnetic beads coupled to goat and anti-mouse Ig (Advanced Magnetics, Inc., Cambridge, MA) these populations were depleted of T cells, B cells, monocytes by negative selection using a magnet. The remaining cells which were enriched for NK cells were phenotypically less than 5% CD3⁺, 75-95% CD56⁺, and 65-80% CD16⁺ as determined by flow cytometry using an Epics profile (Coulter Electronics, Hialeah, FL). These cells were then permeabilized with digitonin as described in Anderson, J. Immunol. 143:1889 (1989). Permeabilized NK cells (50 x 10⁶ cells per ml PBS), were injected into a five week old Balb/c mouse at three week intervals for a total of four immunizations. Three days after the last immunization, the immunized mouse was sacrificed and splenocytes prepared using standard methods. Immune splenocytes were fused to the NS1 hybridoma cell line at a 1:1 ratio using polyethylene glycol as described in Anderson, J. Immunol. 143:1889 (1989). Following fusion, cells were cultured at limiting dilution in a 96-well plate in the presence of RPMI media containing 10% fetal calf serum and HAT selection medium. Individual supernatants were screened for their reactivity with permeabilized and unpermeabilized NK cells, T cells, B cells, monocytes. Monoclonal antibody 5H10 (anti-PEN5) was selected as an antibody which reacted specifically with peripheral blood NK cells.

More specifically, the reactivity pattern of 5H10 was first

induced by mitogenic concentrations of PHA or Con A (in the presence or absence of PMA), nor B cell activation induced by Staphylococcus aureus Cowan strain I, for 1 to 6 days induced the cell surface expression of the PEN5 epitope (See Table 1 below).

5 Similarly, allogeneic T cell clones (CD3⁺CD4⁺ or CD3⁺CD8⁺) did not express the PEN5 epitope (See Table 2).

10 Cell surface expression of the antigen recognized by the antibody 5H10 on hematopoietic cells was also assessed by indirect immunofluorescence and flow cytometry in accordance with established protocols. As summarized in Table 1 below, cell surface staining of monocytes, granulocytes, platelets and erythrocytes also failed to reveal the PEN5 epitope, confirming that PEN5 is an NK cell restricted molecule.

Splenic B cells were activated with optimal mitogenic concentrations of *Staphylococcus aureus* Cowan strain I, and immunofluorescence staining was performed at days 2, 4 and 6 after activation.

Table 2. Absence of surface expression of PEN5 on cytotoxic T cell clones

| Clone | Cell surface expression* | | | | | |
|---------|--------------------------|-----|-----|-----|------|------|
| | CD3 | CD2 | CD4 | CD8 | CD56 | PEN5 |
| T4C1 | + | + | + | - | - | - |
| 6.5 B4 | + | + | + | - | - | - |
| 6.5 C1 | + | + | + | - | - | - |
| 20.1 A2 | + | + | + | - | - | - |
| 8.17 A | + | + | + | - | + | - |
| 20.1 D8 | + | + | - | + | - | - |
| T4T8C1 | + | + | + | - | + | - |

*The cell surface phenotype of the indicated T cell clones was performed by immunofluorescence and flow cytometry. -:5% positive stained cells; +:>60% positive stained cells.

To more precisely analyze the expression of PEN5 on NK cells, flow cytometric analysis of PEN5 expression was performed on

distinct subsets of NK cells, $PEN5^+$ and $PEN5^{dim/-}$ which overlap with the $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets, respectively.

Example 2

$PEN5$ expression is down-regulated by NK cell activation.

5 $CD56^{dim}$ and $CD56^{bright}$ NK cells strongly differ in their response to proliferative stimuli. Although $CD56^{dim}$ NK cells do not proliferate in response to either IL-2 or the combination of ionomycin and PMA, $CD56^{bright}$ NK cells proliferate in response to either stimulus. We took advantage of the recent observation that
10 $CD56^{dim}$ NK cells can be induced to proliferate in response to a combination of LCM and ionomycin to correlate $PEN5$ expression with the NK cell proliferative state. Briefly, sorted $CD56^{dim}$ and $CD56^{bright}$ NK cells were activated for 20 days with ionomycin and LCM as described in the Materials and Methods. At 0, 6, 8, 10, 14, and 20
15 days of culture, aliquots of the activated NK cell populations were analyzed for their cell surface phenotype by flow cytometry using isotype matched control mAb, anti- $CD56$ and 5H10 mAb. The results illustrated in Figure 3 indicate the percent of positively stained cells (%); the total mean fluorescence intensity is indicated below
20 in the histograms.

As shown in Figure 3, activation of $CD56^{dim}$ NK cells resulted in the temporal reduction of $PEN5$ expression. In parallel, the cell surface expression of $CD56$ was temporally increased, and after 20 days of activation, the cell surface expression of $PEN5$ and $CD56$ on
25 the $CD56^{dim}$ NK cells was similar to that of unactivated $CD56^{bright}$ NK cells (i.e: $PEN5^{dim/-}$ and $CD56^{bright}$). These results are consistent

the results of which are shown in Figure 6. In these experiments, detergent lysates prepared from radioiodinated NK cells were immunoprecipitated using 5H10 mAb. Affinity-purified PEN5 α and β glycoproteins were eluted from the antibody-coated sepharose beads using 0.15M NH₄OH, pH 10.5. Aliquots of this dried sample were then subjected to deglycosylation for 24 hr at 37°C using PNGase F (lane 6), O-glycanase (lane 3), keratanase I (lane 2), O-glycanase and keratanase (lane 4), neuraminidase (lane 6), and PNGase F and neuraminidase (lane 7). Control eluates incubated in PBS without any enzymes were separated in lane 1. Samples were separated under non-reducing conditions on a 6-12% SDS-polyacrylamide gradient gel.

Compared to the migration of untreated PEN5 glycoproteins (Figure 6, lane 1), PNGase F treatment induced the disappearance of PEN5 α from the 210-245 kDa m.w. range, and the appearance of a deglycosylated form of PEN5 α migrating at 20-25 kDa (c2). In contrast, the apparent mobility of PEN5 β was reduced by only ~20 kDa after PNGase F incubation. Treatment of PEN5 glycoproteins with O-glycanase (Figure 6, lane 3) did not significantly affect their SDS-PAGE migration pattern. These results indicate that the PEN5 α and PEN5 β differ markedly in their carbohydrates composition, and that ~85% of the apparent m.w. of PEN5 α is due to N-linked carbohydrates.

(Fig. 6, lane 7), resulted in the same effect that PNGase F alone, confirming the presence of terminal sialic acid residues on N-linked carbohydrates present on PEN5 α . The c1 and c2 deglycosylated forms of PEN5 α and β proteins were not immunoprecipitable by the 5H10 mAb (data not shown), indicating that the epitope recognized by the anti-PEN5 mAb requires the keratanase I-sensitive carbohydrate chains.

Example 4

Reactivity of anti-PEN5 mAb with keratan sulfate glycosaminoglycans.

In order to test whether the anti-PEN5 mAb was directed against keratan sulfate carbohydrates, we next examined the effect of exogenous keratan sulfate carbohydrates on the binding of PEN5 mAb to NK cells. Radioiodinated 5H10 (anti-PEN5) mAb was combined with various concentrations of bovine cornea keratan sulfate proteoglycan (BC), and the mixture was then incubated with NK cells.

Briefly, I^{125} -labeled 5H10 mAb (1×10^6 cpm/sample) was preincubated for 20 min at 4°C in PBS in the presence of the concentrations of bovine cornea keratan sulfate (BC) indicated in Figure 7A. The mixture was then added to NK cells for another 20 min incubation at 4°C, prior to three washes in PBS-1%BSA. Samples were counted in a τ -counter, and results were expressed as mean cpm of duplicate samples (SD<10%). When used in incubation with NK cells or anti-PEN5 mAb, the following carbohydrates used at 10 mg/ml were without any effect on 5H10 binding to NK cell surface: chondroitin sulfate B, heparin, heparan sulfate, dextran sulfate,

proteoglycans expressed in various tissues. The antigenicity of 5H10 mAb for aggrecan proteoglycans was analyzed by ELISA as described in *Materials and Methods*. The anti-keratan sulfate mAb 5D4 was used as a positive control. Chondroitinase ABC was used at 0.04 U/ml, 5 keratanase I was used at 0.05 U/ml and keratanase II was used at 0.004 U/ml, for 1 hr at 37°C.

As illustrated in Figure 7C (upper panel), the 5H10 mAb (cross-hatched) recognized aggrecan-type proteoglycans derived from embryonic chick cartilage (CD1, upper panel) and from bovine nasal 10 cartilage (BNC, middle panel). As a positive control, the anti-keratan sulfate mAb 5D4 (open bars) also reacted with untreated CD1 and BNC, whereas its reactivity with keratanase-treated samples was reduced. Treatment of CD1 and BNC with either keratanase I or II, reduced 5H10 reactivity. Treatment of CD1 and BNC with chondroitinase ABC is known 15 to increase the expression of keratan sulfate epitopes. Consequently, digestion of CD1 and BNC with chondroitinase ABC increased the binding of both 5H10 and 5D4. As a negative control, neither mAb recognized the Swarm rat chondrosarcoma aggrecan (RC), which does not contain keratan sulfate (Figure 7C, lower panel). Although 5D4 also reacted 20 with the keratan sulfate proteoglycan isolated from shark cranial cartilage (SHK), 5H10 did not. These results indicate that the 5H10 epitope is present in some, but not all, keratan sulfate chains. Although 5H10 can clearly recognize an epitope expressed on certain keratan sulfate chains, the epitope expressed on the PEN5 molecule on 25 NK cells is not simply a keratan sulfate chain since flow

down-regulates the cell surface expression of the PEN5 epitope. Third, the 5H10 mAb recognizes two distinct aggrecan-type keratan sulfate proteoglycans. Keratan sulfates are glycosaminoglycans consisting of repeated Galb1-4(sulfated)GlcNac disaccharides. Within this constraint, differential branching of the disaccharide subunits, differential sulfation of GlcNac, and differential fucosylation and/or sialylation of the Galb1-4(sulfated)GlcNac can lead to heterogeneity in individual keratan sulfate chains. The lack of reactivity of anti-PEN5 mAb with the keratan-sulfate proteoglycan SHK isolated from shark cranial cartilage suggests that the standard lactosaminoglycan repeat sequence is not the epitope recognized by 5H10. Rather, our data indicate that 5H10 recognizes an unusual sulfated polylactosamine epitope present on some but not all keratan sulfate glycosaminoglycans.

Example 6

Identification of Tissue-Infiltrating Natural Killer Cells Expressing the Mucinlike Glycoprotein PEN51

Materials and Methods

The following methods and materials apply to Examples 6A through 6D.

Source of Tissues. Histologically normal fetal (20 week gestation) and adult human tissues were obtained from surgical and autopsy specimens. Frozen tissues embedded in OCT compound (Baxter Corp., McGaw Park, IL) were stored at -70°C until needed. All tissues were used as frozen tissue sections and were adequately

Chemical Co., St. Louis, MO) reconstituted in purified water. Frozen sections were cryostat cut (6-8 mm thick), collected onto coated slides, air dried and fixed in 2% neutral buffered paraformaldehyde at 4°C for 20 minutes, followed by several washes with PBS. To block endogenous biotin content, and reduce cross-reactivity of the biotinylated antibody, all tissues were incubated with a solution of avidin (Vector) and 10% normal horse serum (Vector) in BSA dilution buffer, at room temperature for 15 minutes. Tissue sections were drained of avidin/horse serum buffer and incubated with the antibody at 4°C, overnight. After washing in PBS, slides were incubated for 30 minutes in 0.3% hydrogen peroxide and biotin blocking solution to quench endogenous peroxidase activity and to block remaining avidin. Sections were then washed with PBS, incubated with either biotinylated goat anti-mouse IgM or horse anti-mouse IgG antibodies for 30 minutes, washed in PBS, incubated with avidin-biotin-peroxidase complexes for 45 minutes, and then washed again with PBS. After incubating the slides for 5 minutes in Tris-Imidazole/HCL buffer, the peroxidase reaction was initiated by incubating for 5 minutes with 3,3-diaminobenzidine (DAB) (Sigma Chemical Co.) dissolved in Tris-Imidazole/HCL buffer containing 0.11% hydrogen peroxide. Tissue sections were washed in water, counterstained with Harris hematoxylin, and dehydrated through graded alcohols and xylenes. Coverslips were then mounted on slides with E-Z-Mount mounting media (Shandon Inc., Pittsburgh, PA).

Transmission Electron Microscopy. Peripheral blood NK cells

chromatin was dense and homogeneous. Immunostaining was usually in the region of the plasma membrane, but was also seen in the cytoplasm.

5 **Example 6B:**

Comparative expression of PEN5⁺ and CD56⁺ lymphocytes in fetal and adult tissues.

Because fetal liver and fetal thymus have been implicated as sites of NK cell differentiation, we compared the expression of
10 PEN5⁺ and CD56⁺ lymphocytes in each of these tissues to that of their adult counterparts. As shown in Figure 10, CD56⁺ cells were not easily detected in either fetal or adult thymus. In each of these tissues, scattered lymphocytes expressing low levels of CD56
15 could be detected at high magnification, suggesting that CD56⁺ cells are present, but difficult to detect using this histochemical method. This might result from lability of the antigen under these fixation conditions, or the low level of CD56 expression, since Sanchez, et al [J. Exp. Med. 178:1857 (1993)] have shown that CD56⁺ lymphocytes can be identified in these tissues using flow
20 cytometric analysis. In contrast, PEN5⁺ cells were easily detected, scattered throughout both adult and fetal thymus. The density of PEN5⁺ cells was consistently greater in fetal thymus than in adult thymus. Occasional CD56⁺ cells could be detected in adult liver, but again, the intensity of staining was very weak
25 (Figure 11). Scattered PEN5⁺ cells were easily detected in the adult liver, due to their more intense staining. Relatively more

presence of IL-2 results in the differentiation of cells which phenotypically and functionally resemble peripheral blood NK cells [Sanchez, et al., J. Exp. Med. 178:1857 (1993); Koyasu, et al., J. Exp. Med. 179:1957 (1994); Michon, et al., J. Immun. 140:3660 (1988); and Mingari, et al., J. Exp. Med. 174:21 (1991)]. Some of these studies rely on the characterization of lymphocyte clones that grow out of selected fetal and adult tissues. As clonal selection may impart a bias on any analysis of cell populations, the observation that PEN5⁺ lymphocytes are present in fetal liver and thymus provides unbiased evidence for the differentiation of NK cells in these tissues. Although relatively few CD56⁺ cells were identified at these sites using histochemical analysis, this result might reflect the low density of expression of this NK marker. CD56⁺ lymphocytes have been detected in both fetal liver and fetal thymus using flow cytometric analysis [Sanchez, et al., J. Exp. Med. 178:1857 (1993)]. Our results suggest that PEN5 expression can be expected to be a more sensitive marker of tissue infiltrating NK cells than CD56 expression.

Example 6C:

Expression of PEN5 antigen on non-lymphoid cells.

Antibodies reactive with PEN5 also recognized some non-leukocytic cells. These were generally epithelial cells found in the esophagus, cervix, endometrium, trachea, bile ducts, colon and pancreas. The most dramatic example of this non-lymphoid staining was seen in the lung and colon, where anti-PEN5 strongly

the expression of TIA-1 that are related to NK cell differentiation. Table III tabulates the percentage of PEN5⁺ tissue infiltrating lymphocytes expressing TIA-1 in several tissues. As summarized below, whereas the majority of PEN5⁺ lymphocytes co-express TIA-1 in spleen and liver, this is not the case in tonsil or appendix, where most PEN5⁺ lymphocytes do not express TIA-1. Whether these tissue specific differences reflect different stages of NK cell differentiation, or different types of tissue infiltrating lymphocyte remains to be elucidated.

Taken together, the results provided in Examples 6A through 6D illustrate a number of important findings. We have used a monoclonal antibody reactive with a sulfated poly-N-lactosamine epitope expressed on the NK cell restricted glycoprotein PEN5 to survey the presence of tissue-infiltrating NK cells in lymphoid and non-lymphoid tissues. Whereas antibodies reactive with CD56 were unable to efficiently detect all tissue infiltrating NK cells, PEN5⁺ lymphocytes were readily identified in multiple tissues. Assuming that PEN5 is expressed similarly on both tissue infiltrating and circulating lymphoid cells, these results suggest that NK cells can infiltrate multiple lymphoid and non-lymphoid tissues to mediate their immune functions. In the periphery, PEN5 is selectively expressed on large granular lymphocytes possessing cytotoxic effector function. These cells express low levels of CD56, which might account for the inability of antibodies reactive with CD56 to recognize these cells in tissues. Double staining with the cytotoxic granule marker, TIA-1, supports the conclusion that PEN5⁺ lymphocytes infiltrating some tissues (e.g. spleen and liver) contain cytotoxic granules. Surprisingly, however, many PEN5⁺ cells infiltrating other tissues (e.g. tonsil and appendix) did not co-express TIA-1. This result suggests that in some tissues, PEN5 might be expressed on agranular lymphocytes.

The PEN5 epitope recognized by monoclonal antibody 5H10 is related to keratan sulfate, which is itself a member of the polylactosamine family of sugars. The two isoforms of PEN5 thus resemble a keratan sulfate proteoglycan (PEN5 β) and a keratan

Having described the invention, what is claimed is:

1. A monoclonal antibody that identifies a unique epitope present on a natural killer cell-surface glycoprotein pair designated PEN5 and comprising PEN5 α and PEN5 β , which epitope is preferentially expressed on natural killer cells having the phenotype CD16⁺ CD56^{dim} relative to natural killer cells having the phenotype CD16⁺ CD56^{bright} and is not present on CD3⁺ T cells or CD20⁺ B cells.
2. An antibody according to claim 1, which is a mouse monoclonal antibody.
3. An antibody according to claim 1, which recognizes an epitope that is not present on monocytes, granulocytes, platelets, or red blood cells.
4. An antibody according to claim 1, which is a chimeric antibody.
5. An antibody according to claim 1, in which the epitope is a sulfated polylactosamine carbohydrate.
6. An antibody according to claim 1, which binds the same epitope as the antibody secreted by the hybridoma identified by ATCC Accession No. HB11441.
7. An antibody according to claim 1 that is produced by a hybridoma identified by ATCC Accession No. HB11441
8. An immunoreactive fragment of an antibody according to claim 1.
9. An immunoreactive fragment of an antibody according to claim 2.

phenotype CD16⁺ CD56^{bright} and is not present on CD3⁺ T cells or CD20⁺ B cells, or a fragment or derivative of the antibody, and

(b) detecting immune complex formation.

17. A method according to claim 16, wherein the monoclonal antibody is attached to a label.

18. A method according to claim 17, wherein the monoclonal antibody is labeled with a detectable label selected from the group consisting of a radioisotope, a fluorescent label, or an enzyme.

19. A method according to claim 18, wherein the mixed population of cells is contained in a sample of peripheral blood or bone marrow.

20. A method according to claim 19, wherein the label is a fluorescent label selected from the group consisting of rhodamine, phycoerythrin, and fluorescein isothiocyanate.

21. A method for selectively eliminating CD16⁺, CD56^{dia} natural killer cells from a sample comprising a mixed population of cells comprising the steps of:

(a) contacting the sample with a monoclonal antibody that identifies a unique epitope present on a molecule consisting essentially of a pair of glycoproteins, PEN5 α and PEN5 β , which epitope is preferentially expressed on natural killer cells having the phenotype CD16⁺ CD56^{dia} relative to natural killer cells having the phenotype CD16⁺ CD56^{bright} and is not present on CD3⁺ T cells or CD20⁺ B cells, or an immunoreactive fragment or derivative of the antibody, and

(b) removing from the sample the cells that bind to the

identified by ATCC Accession No. HB11441.

28. A kit according to claim 27, wherein the monoclonal antibody is attached to a label selected from the group consisting of rhodamine, fluorescein isothiocyanate, phycoerythrin, and biotin.

29. A kit according to claim 28, further comprising an antibody that recognizes a T cell surface antigen, CD3, and an antibody that recognizes a B cell surface antigen, CD20.

30. An antibody according to claim 1, wherein the epitope is expressed on about 70 to 90% of natural killer cells that have the phenotype CD16⁺ CD56^{dim} but only on about 10 to 35% of natural killer cells that have the phenotype CD16⁺ CD56^{bright}.

2/15

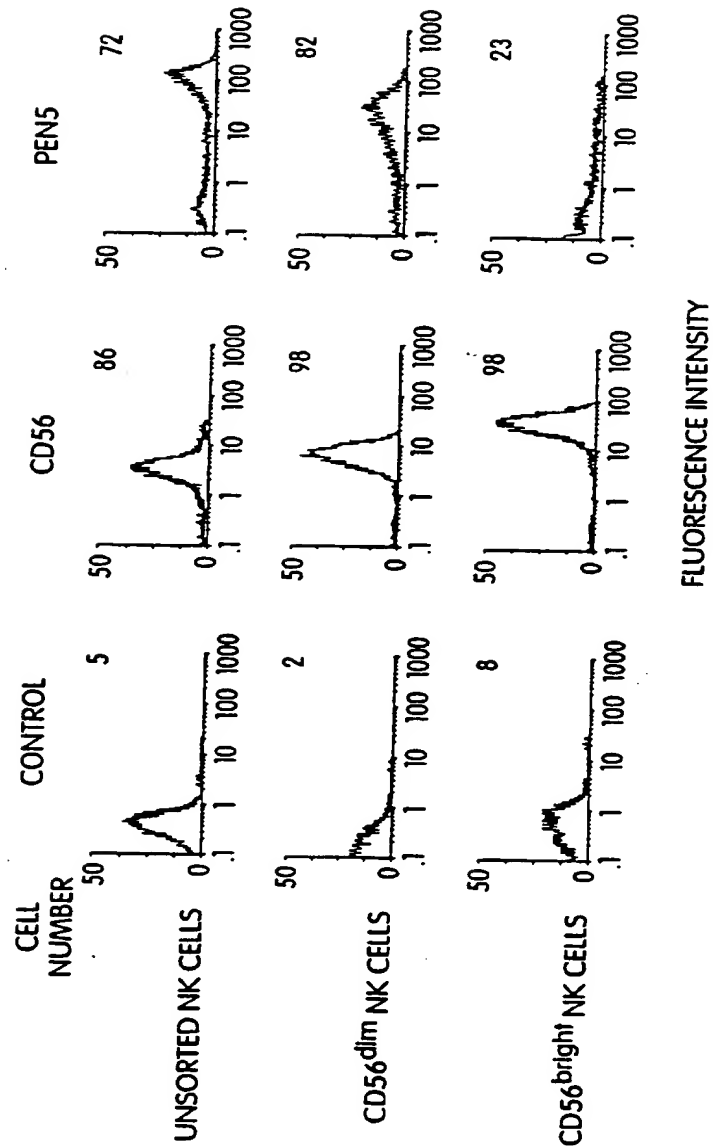


Fig. 2

4/15

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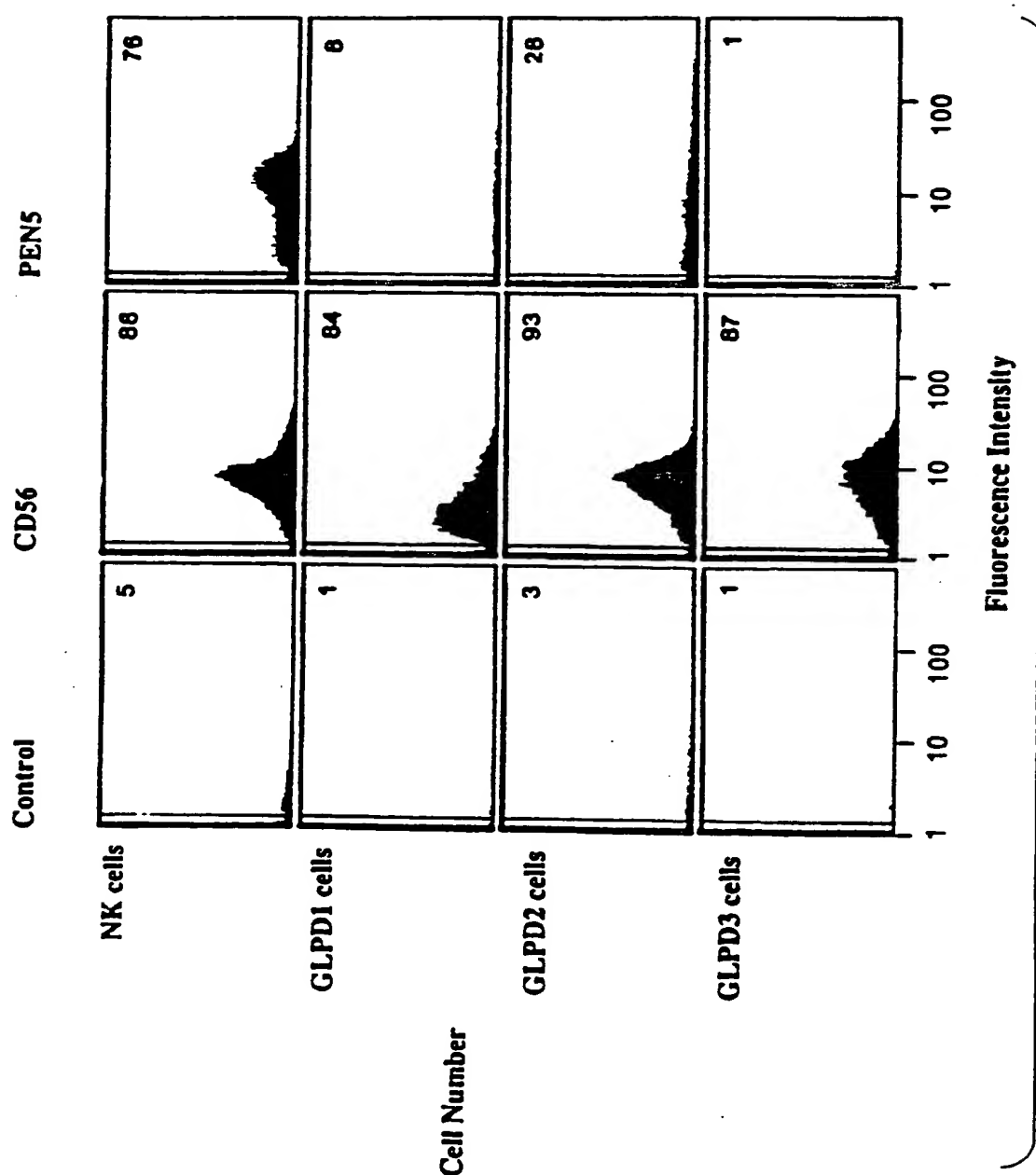


Fig. 4

6/15

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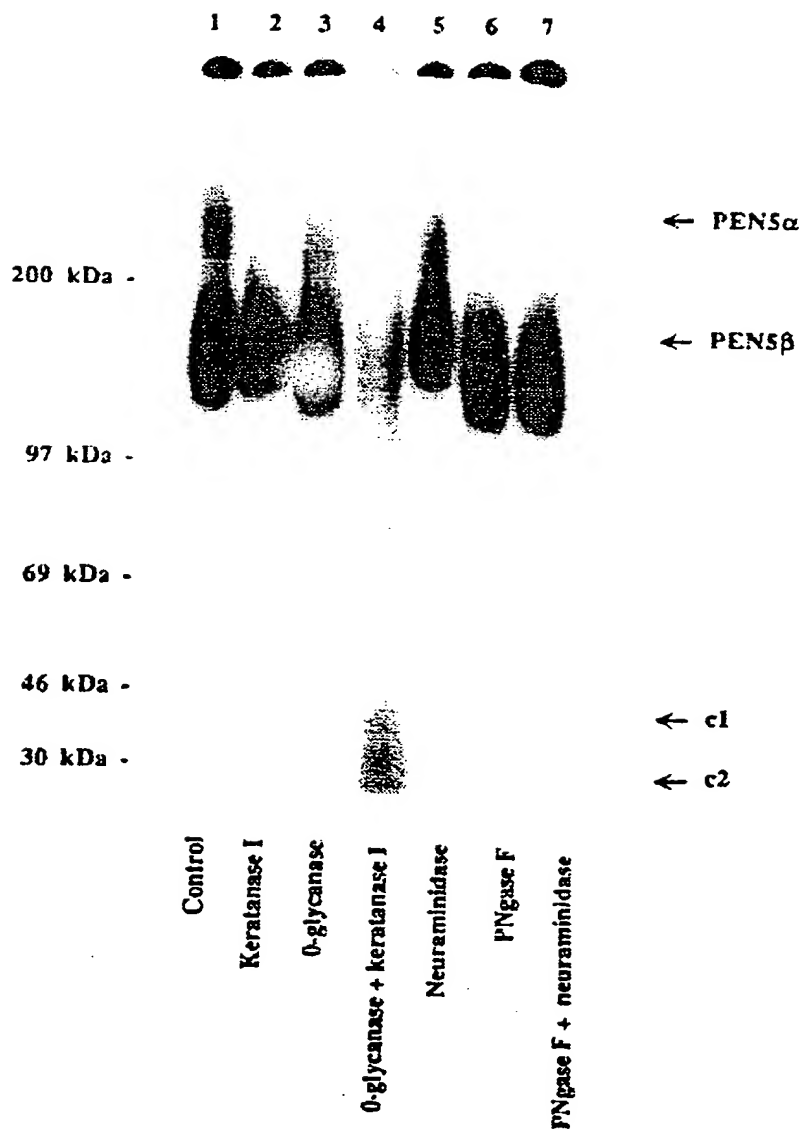


Fig. 6

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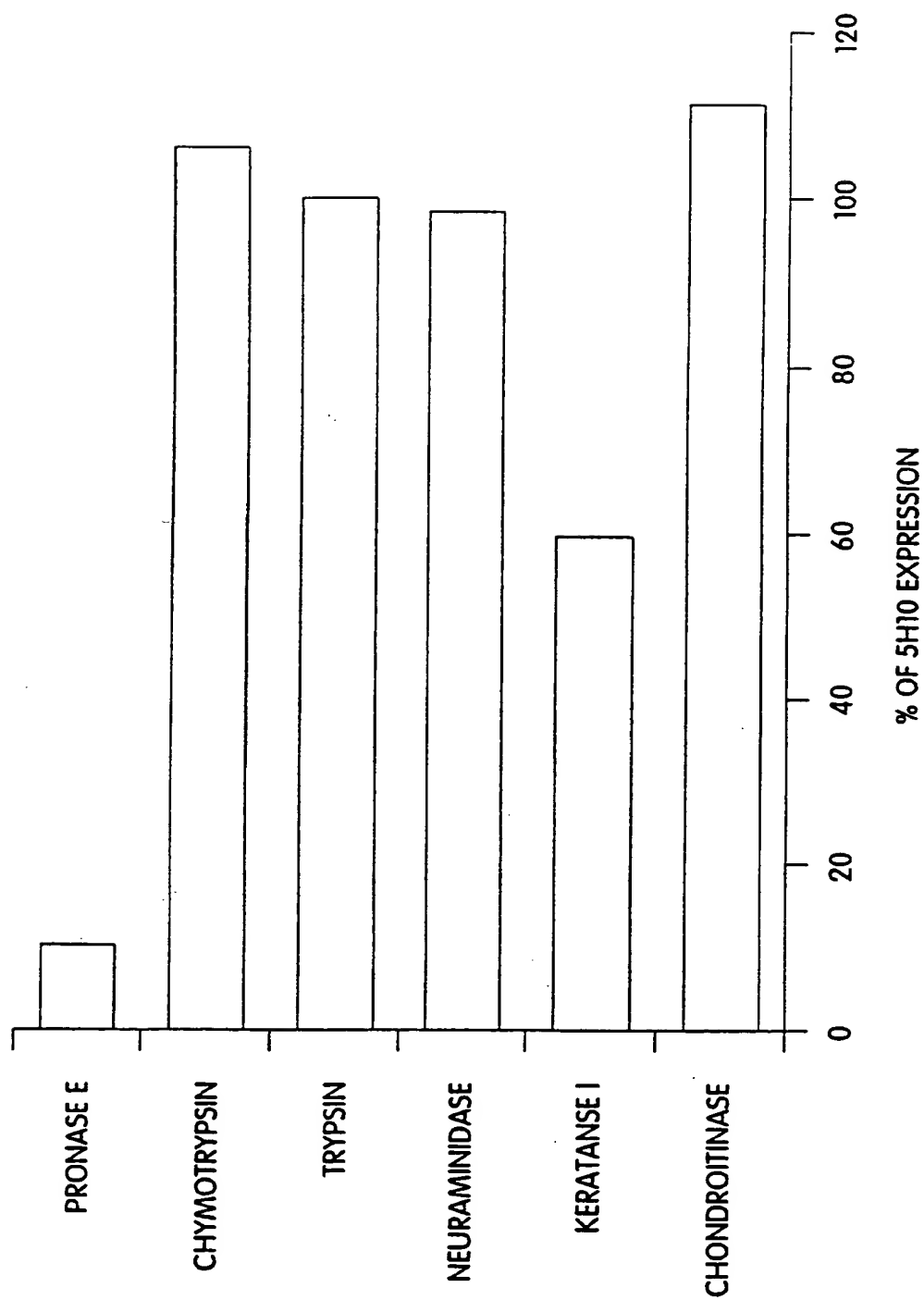


Fig. 7B

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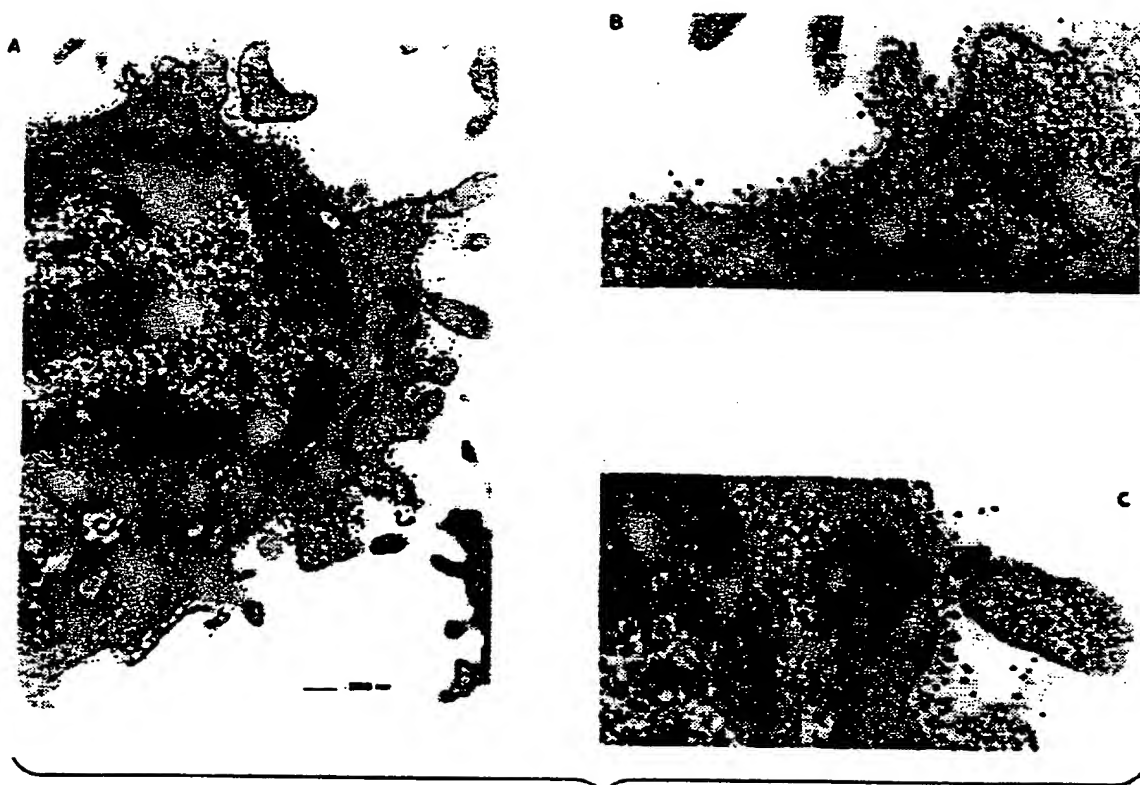


Fig. 8

12/15

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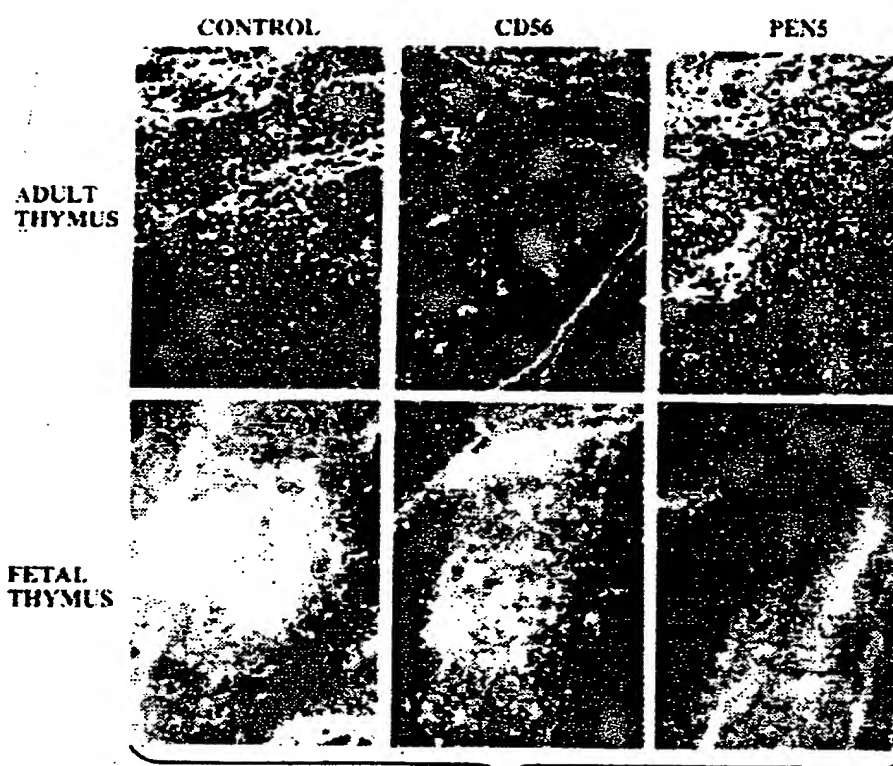


Fig. 10

14/15

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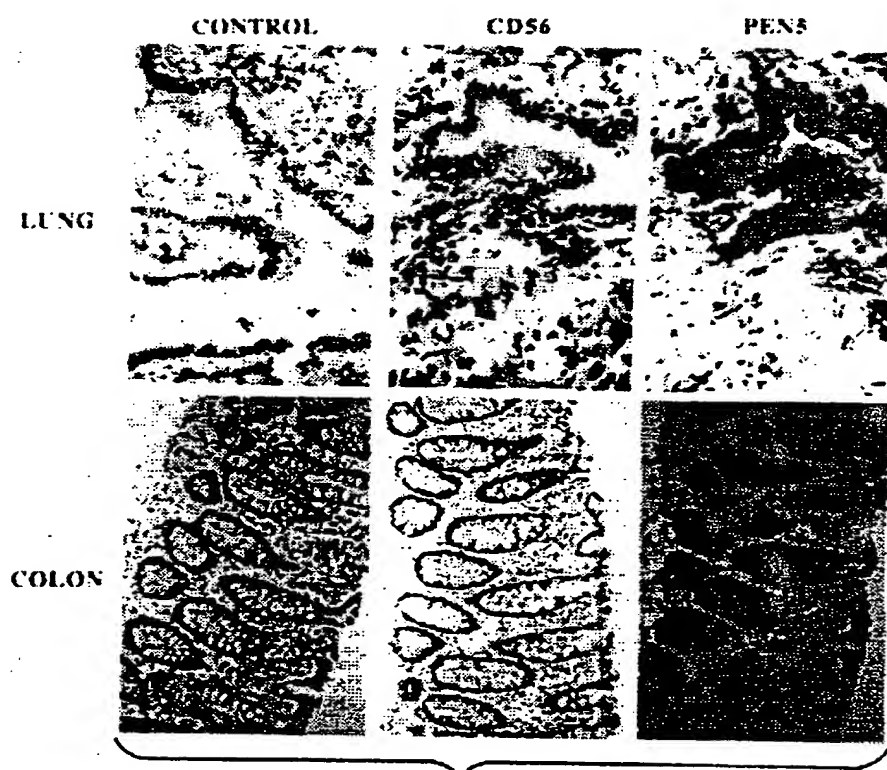


Fig. 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/09714

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|--|-----------------------|
| Y | US, A, 4, 831,122 (BUCHSBAUM ET AL.) 16 May 1989, see column 1, line 49 to column 3, line 2; columns 3-4; column 9, line 38 to column 10, line 56. | 11, 21, 22, 24 |
| Y | US, A, 5,068,223 (LIPSKY ET AL.) 26 November 1991, see column 4, lines 25-27. | 11 |
| Y | US, A, 5,215,927 (BERENSON ET AL.) 01 June 1993, see column 2, lines 4-56. | 13 |
| Y | US, A, 4,797,475 (TERASAKI ET AL.) 10 January 1989, see column 3, line 30 to column 6, line 65. | 21, 22, 23 |

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/09714

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

G01N 33/48, 33/483, 33/49, 33/50, 33/53, 33/537, 33/543, 33/563, 33/577, 33/58; C07K 16/18, 16/28, 16/46; C12N 5/12

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

Group I, Claims 1-10, 12-20, and 26-30, drawn to a monoclonal antibody specific for a natural killer cell surface glycoprotein, a hybridoma which produces this monoclonal antibody, kits containing this monoclonal antibody, and a method for detecting natural killer cells using the monoclonal antibody.

Group II, Claims 11 and 21-24, drawn to toxin-labeled monoclonal antibody specific for a natural killer cell surface glycoprotein and methods for selectively eliminating natural killer cells from mixed cell population using this monoclonal antibody.

Group III, Claim 25, drawn to an isolated natural killer cell-specific cell surface glycoprotein.

The subcombinations as claimed lack unity of invention under PCT Rule 13.2 since they each recite a special technical feature which is not recited in the other subcombinations. The claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept. The invention of Group I is drawn to an antibody, kits, and methods for natural killer cell detection while the invention of Group II is drawn to methods for eliminating natural killer cells, and the method steps of group I are materially different from the method steps of Group II. Group III is drawn to an isolated glycoprotein found on natural killer cell surfaces. The isolated protein of Group III is not required for the methods of Groups I and II.